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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Edge	Examiner:	Decloux, Amy M
Serial No.:	09/624,885	Art Unit:	1644
Filing Date:	July 24, 2000		
Title:	MUSCLE CELLS AND THEIR USE IN CARDIAC REPAIR		

Mail Stop AF
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Jonathan Dinsmore, declare as follows:

1. I am the Senior Director for Cell Transplantation Research at Diacrin, Inc. I have held this position for 11 years. My curriculum vitae is attached.

2. I am well familiar with the field of tissue culture, and particularly with the culture of myogenic cells.

3. I have reviewed and am familiar with the specification of United States Patent Application No. 09/624,885 (the '855 application) for "Muscle Cells and Their Use in Cardiac Repair" by Albert Edge. I have also reviewed the currently-pending claims in this application, and understand them to encompass compositions of isolated skeletal myoblasts and isolated fibroblasts, so long as the compositions are substantially free of

myotubes. I further understand that some of the claims specifically recite compositions of human cells.

4. I have read the Office Action in the '885 application, mailed on March 25, 2003, and understand that the Examiner has asserted 1) that a scientist of ordinary skill would not understand the '885 application to describe cell compositions that are substantially free of myotubes; and 2) that the '885 application does not describe compositions of human cells. I understand that the Examiner has specifically stated that the '885 does not provide any example of the preparation of a composition of human cells that contains skeletal myoblasts and fibroblasts but is substantially free of myotubes. I disagree with the Examiner's assertions.

5. The purpose of this Declaration is to establish 1) that a scientist of ordinary skill, reading the '885 application, would understand it to describe cell populations that are substantially free of myotubes; 2) that a scientist of ordinary skill, reading the present specification, would understand it to describe preparations of human cells, and particularly to describe preparations that are substantially free of myotubes; and 3) the preparations of human cells described in the '885 application are in fact substantially free of myotubes.

6. To address the first point first, I can attest as a scientist in the field that the '885 application clearly and specifically indicates a preference for compositions that are substantially (or even completely) free of myotubes. The entire purpose of the invention is to provide compositions that will form myotubes *in situ* after the composition is injected into heart tissue. Any person of ordinary skill would understand that it is desirable for the compositions to be substantially free of myotubes prior to their injection. This fact is confirmed throughout the specification. For example:

a. The very definition of a skeletal myoblast that is given in the '885 application emphasizes that what is desired in the inventive compositions are *precursor* cells (see, for example, page 5, line 20) and not mature myotubes.

b. Furthermore, at several points, the '885 application indicates that it is desirable to characterize the inventive compositions by FACS analysis (see, for example, page 9, lines 26-29). Myotubes, if they are present in a sample that is submitted for FACS analysis, prevent FACS analysis of the cells in the sample because they are so large they occlude the scanner's opening aperature and stop the scan. Thus, if successful FACS analysis is performed on a sample, it can be concluded that the sample was substantially free of myotubes. A skilled scientist, reading in the '885 specification that inventive compositions could or should be characterized by FACS, would understand that compositions that are substantially free of myotubes are desired.

c. Furthermore, throughout the '885 application, there is continued emphasis on limiting doubling times (see, for example, page 8, line 27-page 9, line 5) in order to avoid cell maturation. For all of these reasons, the '885 application clearly informs the skilled scientist that cell preparations substantially free of myotubes are desired.

7. With respect to the second and third points, I note that, as one aspect of my work at Diacrin, I am responsible for overseeing the processing of human tissue into skeletal myoblast compositions. This processing is performed in accordance with the description in the '885 application, as follows:

a. The process is initiated when a piece of human skeletal muscle is shipped to

Diacrin. That muscle is subjected to enzymatic digestion to liberate the myoblasts from the intact muscle fiber as is discussed in the '885 application, for example, at page 8, lines 18-23. This digestion step is performed under conditions designed to maximize myoblast purity, as indicated in the '885 application (see, for example, page 8, lines 25-26).

b. The isolated cells are plated onto a tissue culture surface with growth medium, such as, for example, as described at page 10, line 12- page 11, line 2 or page 11, lines 7-10, of the '885 application. Cells are allowed to expand to a desired number, and are tested periodically by FACS analysis, as described in the '885 application (see, for example, page 9, lines 26-29) to determine the percentage of myoblasts in the population. Typically, the expansion period lasts for about 11-13 doublings, within the range specified in the '885 application. For example, the attached Exhibit A shows a FACS assay data record for a particular culture of human cells during expansion. As noted above, the successful determination of myoblast and fibroblast cell numbers by FACS analysis provides evidence that the compositions were substantially free of myotubes.

c. After the culture period, the cell populations are again FACS sorted to assess the relative percentages of myoblast and fibroblast cells. Before they are released for implantation, the cell populations are tested to ensure that they contain functional skeletal myoblasts (i.e., cells having the ability to fuse and form myotubes). For example, the attached Exhibits B and C show, respectively, the FACS and fusion assay results for the final preparation of cells whose culture was assayed in Exhibit A. Once again, the FACS results demonstrate that the compositions are substantially free of myotubes. As further confirmation of this fact, no myotubes

are observed in the fusion assay for at least 2-3 days.

8. I, Jonathan Dinsmore, declare that all statements made herein of my own knowledge are true and that these statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patents that may issue thereon.

Respectfully Submitted,

Jonathan Dinsmore

Name: Jonathan Dinsmore
Title: Sr. Director of Cell Transplantation
Date: 5/27/03

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Professional Experience

Senior Director of Cell Transplantation Research, 3/99 - present
Diacrin, Inc., Charlestown, MA
Director of Cell Transplantation Research, 1/95 – 3/99
Diacrin, Inc., Charlestown, MA
Principal Investigator, 1/93 – 1/95
Diacrin, Inc., Charlestown, MA
Research Scientist, 7/92 – 1/93
Diacrin, Inc., Charlestown MA

Education

Massachusetts Institute of Technology, Cambridge, MA
Post-doctoral fellow. Research Topic: "Alteration of gene expression during development with antisense RNA expression." Center for Cancer Research and Department of Biology. Laboratory of Dr. Frank Solomon, 7/89 – 6/92.
Dartmouth College, Hanover, NH
Ph. D. Biology. Thesis Title: "Biochemistry of the isolated mitotic apparatus." Awards/Honors: Cass Traveling Fellowship Award 1985, Presidential Scholar 1988. Ph. D. awarded 6/89
Boston College, Chestnut Hill, MA
B. S. Biology, Cum Laude, Graduation 6/83

Additional Professional Activities

United States Antarctic Research Program, Palmer Island, Antarctica, Research Student. "Biochemical analysis of tubulin proteins from Antarctic ice fish."(1/86 – 4/86)

Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, Research student "Advanced Techniques in Molecular Biology." (6/87 – 7/87).

Woods Hole Marine Biological Laboratories, Woods Hole, MA, Research student. "Biochemistry of the isolated mitotic apparatus."(6/85 – 9/85).

University of Washington, Friday Harbor Labs, Research student. "Invertebrate Zoology and Invertebrate Embryology."(6/84 – 9/84).

Membership in Professional Associations: American Society for Cell Biology, New York Academy of Sciences, American Society for Microbiology, Society for Neuroscience, American Society for Neural Cell Transplantation, American Heart Association.

Patents and Patent Applications

Issued Patents:

U. S. Pat. Number 6,432,711: "Embryonic stem cells capable of differentiating into desired cell lines."

U. S. Pat. Number 6,294,383: "Porcine neural cells and their use in the treatment of neurological deficits due to neurodegenerative diseases."

U. S. Pat. Number 6,277,372: "Porcine neural cells and their use in the treatment of neurological deficits due to neurodegenerative diseases."

U. S. Pat. Number 6,258,353: "Porcine neural cells and their use in the treatment of neurological deficits due to neurodegenerative diseases."

U. S. Pat. Number 6,204,053: "Porcine cortical cells and their use in treatment of neurological deficits due to neurodegenerative diseases."

U. S. Pat. Number 6,140,116: "Isolated and modified porcine cerebral cortical cells."

U. S. Pat. Number 5,961,972: "Isolated porcine pancreatic cells for use in treatment of diseases characterized by insufficient insulin activity."

U. S. Pat. Number 5,919,449: "Porcine cardiomyocytes and their use in treatment of insufficient cardiac function."

U. S. Pat. Number 6,491,912: "Porcine cardiomyocytes and their use in treatment of insufficient cardiac function."

U. S. Pat. Number 5,837,236: "Isolated porcine pancreatic cells for use in treatment of diseases characterized by insufficient insulin activity."

U. S. Pat. Number 5,677,174: "Isolated porcine pancreatic cells for use in treatment of diseases characterized by insufficient insulin activity."

U. S. Pat. Number 5,629,194: "Isolated porcine pancreatic cells for use in treatment of diseases characterized by insufficient insulin activity."

U. S. Pat. Number 5,593,673: "Isolated porcine pancreatic cells for use in treatment of diseases characterized by insufficient insulin activity."

U. S. Pat. Number 6,444,205: "Transplantation of neural cells for the treatment of chronic pain or spasticity."

Pending Applications:

U. S. Application Number 09/110,772	"Improved methods for storing neural cells such that they are suitable for transplantation."
U. S. Application Number 09/163,272	"Porcine spinal cord cells and their use in spinal cord repair."
U. S. Application Number 09/163,227	"Transplantation of neural cells for the treatment of ischemic damage due to stroke."

Research Papers

(1) Dinsmore, J. H. and R. D. Sloboda. (1988). Calcium and calmodulin-dependent phosphorylation of a 62kD protein induces microtubule depolymerization in sea urchin mitotic apparatuses. **Cell 53:769-780.**

(2) Dinsmore, J. H. and R. D. Sloboda. (1989). Microinjection of antibodies to a 62kd mitotic apparatus protein arrests mitosis in dividing sea urchin embryos. **Cell 57:127-134.**

(3) Dinsmore, J. H. and R. D. Sloboda. (1989). Identification of a 62kD mitotic apparatus associated protein from sea urchin which is important for the proper progression of mitosis. **Ann. N.Y. Acad Sci. 582:301-303.**

(4) Dinsmore, J. H. and F. Solomon. (1991). Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. **Cell 64: 817-826.**

(5) Birgbauer, E., J. H. Dinsmore, B. Winckler, A. D. Lander, and F. Solomon. (1991). Association of ezrin isoforms with the neuronal cytoskeleton. **J. Neurosci. Res. 30: 232-241.**

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(7) Detrich H. W., T.J. Fitzgerald, J. H. Dinsmore, and S. P. Marchese-Ragona (1992). Brain and egg tubulins from Antarctic fishes are functionally and structurally distinct. **J. Biol. Chem. 267: 18766-18775.**

(8) Dinsmore, J. H. and F. Solomon. (1993). The use of antisense RNA to inhibit expression of cytoskeletal proteins in P19 embryonal carcinoma cells. **Neuroprotocols 2: 19-23.**

(9) Deacon, T. W., P. Pakzaban, L. H. Burns, J. Dinsmore, and O. Isacson. (1994). Cytoarchitectonic development, axon-glia relationships, and long distance axon growth of porcine striatal xenografts in rats. **Exp. Neurol. 130: 151-167.**

(10) Pakzaban, P., T. W. Deacon, L. H. Burns, J. Dinsmore, and O. Isacson. (1995). A novel mode of immunosuppression of neural xenotransplants: masking of donor major histocompatibility complex class I enhances transplant survival in the CNS. **Neuroscience 65: 983-996.**

(11) Garcia, A. R., T. W. Deacon, J. Dinsmore, and O. Isacson. (1995). Extensive axonal and glial fiber growth from fetal porcine cortical xenografts in the adult rat cortex. **Cell Transplantation 4: 515-527.**

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(13) Dinsmore, J. H., J. Ratliff, T. H. Deacon, P. Pakzaban, D. Jacoby, W. Galpern, and O. Isacson. (1996). Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. **Cell Transplantation 5:131-143.**

(14) Dinsmore, J., P. Pakzaban, T. Deacon, L. Burns, W. Galpern, and O. Isacson. (1996). Long-term survival of F(ab')2 masked xenogeneic fetal porcine neural cells after transplantation into brain. **Transplantation Proc. 28:817-818.**

(15) Galpern, W. R., L. H. Burns, T. W. Deacon, J. Dinsmore, and O. Isacson. (1996). Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. **Exp. Neurol. 140:1-13.**

(16) Deacon, T., J. Schumacher, J. Dinsmore, C. Thomas, P. Palmer, S. Kott, A. Edge, D. Penney, S. Kassissieh, P. Dempsey, and O. Isacson. (1997). Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. **Nature Medecine 3:350-353.**

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(24) Jacoby, D., C. Lindberg, J. Pope, J. Ratliff, M. Cunningham, and J. Dinsmore. (1999). Long-term survival of fetal porcine lateral ganglionic eminence cells in the hippocampus of rats. **J. Neurosci. Res. 56: 581-594.**

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Reviews and Book Chapters

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(2) Edge, A. S. B. and J. Dinsmore. 1997. Xenotransplantation in the central nervous system. Xeno 5:23-25.

(3) Dinsmore, J. H. (1998) Treatment of neurodegenerative diseases with neural cell transplantation. Exp. Opin. Invest. Drugs 7:527-534.

(4) Edge, A. S. B., M. Gosse, and J. Dinsmore. 1998. Xenogeneic cell therapy: current progress and future developments of porcine cell transplantation. Cell Transplantation 7: 525-539.

(5) Dinsmore, J. H., J. Martin, J. Siegan, J. P. Morrison, C. Lindberg, J. Ratliff, and D. J. Jacoby. (2002). CNS grafts for treatment of neurologic disorders. Methods in Tissue Engineering. A. Atala, R. P. Lanza (eds.), Academic Press, San Diego, CA, pp.1127-1134, 2002.

Abstracts

(1) Detrich, H. W., T. J. Fitzgerald, M. Little, J. H. Dinsmore, and R. F. Ludueña. 1987. Assembly and structure of brain and egg tubulins from Antarctic fishes. J. Cell Biol. 105: 278a.

(2) Dinsmore, J. H. and R. D. Sloboda. 1987. Identification of a calcium-calmodulin dependent protein kinase associated with the sea urchin mitotic apparatus. Biol. Bull. 173:564 -565.

(3) Dinsmore, J. H. and R. D. Sloboda. 1987. Identification of a calcium/calmodulin dependent protein kinase associated with the sea urchin mitotic apparatus. J. Cell Biol. 105: 284a.

(4) Dinsmore, J. H. and R. D. Sloboda. 1988. Microinjection of antibodies to a 62kD protein from sea urchin mitotic apparatuses inhibits mitosis in dividing sea urchin embryos. J. Cell Biol. 107:443a.

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(9) Birgbauer, E., B. Winckler, J. H. Dinsmore, M. Magendantz, and F. Solomon. 1990. Developmental changes of ezrin association with the cytoskeleton. J. Cell Biol. 111: 423a.

(10) Dinsmore, J. H., D. B., Jacoby and J. Ratliff. 1993. Controlled differentiation of embryonic stem cells in vitro. 33rd Annual Meeting of the American Society for Cell Biology, Dec. 11-15, 1993.

(11) Deacon, T., P. Pakzaban, J. Dinsmore, L. Burns, and O. Isacson. 1993. Axonal growth by fetal porcine striatal grafts in rats. 23rd Annual Meeting of Society for Neuroscience, Nov. 7-12, 1993.

(12) Burns, L. H., P. Pakzaban, T. W. Deacon, J. Dinsmore, and O. Isacson. 1994. Xenotransplantation of porcine ventral mesencephalic neuroblasts restores function in primates with chronic MPTP-induced Parkinsonism. 24th Annual Meeting, Society for Neuroscience. Vol. 20: 1330.

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(15) Dinsmore, J., P. Pakzaban, T. Deacon, L. Burns, and O. Isacson. 1994. Long term survival of masked xenogeneic fetal porcine neural grafts. IBC Conference on Xenotransplantation. 16 - 17 June 1994.

(16) Dinsmore, J. H., P. Pakzaban, T. W. Deacon, J. Ratliff, D. M. Frim, and O. Isacson. 1994. Intracerebral transplantation of neurons differentiated in vitro from pluripotent embryonic stem cells. 24th Annual Meeting of the Society for Neuroscience, Nov 13-18, 1994.

(17) Isacson, O., T. W. Deacon, P. Pakzaban, L. Burns, W. Galpern, J. Dinsmore, S. Tatter, C. LeBlanc, and J. Park. 1994. Long distance graft growth of astroglial fibers is associated with axonal white matter tracts but not axonal gray matter target zones. 24th Annual Meeting. Society for Neuroscience. Vol. 20: 9.

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(20) Pakzaban, P., T. W. Deacon, L. H. Burns, J. Dinsmore, S. Chappel, and O. Isacson. 1994. The use of porcine fetal neuroblasts as an alternative cell source for neural transplantation. 1st Annual Meeting of the American Society for Neural Transplantation, May 5-7, 1994.

(21) Dinsmore, J. H., T. Deacon, P. Pakzaban, J. Ratliff, and O. Isacson. 1995. Neurons differentiated in vitro from pluripotent embryonic stem cells for CNS transplantation: in vitro characterization and transplantation into rodents. 2nd Annual Meeting of the American Society for Neural Transplantation. 27-30 April 1995.

(22) Galpern, W. R., L. H. Burns, T. W. Deacon, S. B. Tatter, J. Dinsmore, and O. Isacson. 1995. Xenotransplantation and antigen masking of fetal porcine ventral mesencephalon in a rat model of Parkinson's disease. 2nd Annual Meeting of the American Society for Neural Transplantation. 27-30 April 1995.

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FACs Assay Data Record

Item	Lot No.	Expiration Date	Manufacturing Initials and Date
Bovine Serum Albumin, P/N	1082046	7/01	M 8/13/01
DPBS P/N	108353	8/03	M 8/13/01
5.1H11 Antibody	5/18/92	TBD.	M 8/13/01
Biotinylated GAM	L0602	TBD	M 8/13/01
Ultra Avidin-FITC, P/N	066L235	TBD	M 8/13/01
1967	M05895	TBD	M 8/13/01

Final Product Lot No.	% of Cells Staining Positive for 5.1H11 Stain by FACS	Operator Initials and Date	
Myo29 P2	84.19	M 8/6/01	
1st batch Myo29 P3	81.7	M 8/10/01	(MY029-05100 Bulk)
2nd batch Myo29 P4	62.7	M 8/14/01	(MY029-08130 Bulk)
1/29/03 2nd batch Myo29 P4	61.3	M 8/21/01	(MY029-08200 Bulk)
2/13/04 batch Myo29 P4	63.2	M 8/21/01	(MY029-08210 Bulk)

Comments: N/A 8/24/01

Reviewed By: [signature] Date: 8/24/01

QF271-0
Effective Date: JUN 0 6 2000

Exhibit A

DIACRIN, INC.

FACS Assay Data Record

Applicable SOP: DQ140

Item	Lot No.	Expiration Date	Manufacturing Initials and Date
Bovine Serum Albumin, P/N	1082046	7/01	KM 8/21/01
DPBS P/N	1083531	8/03	KM 8/21/01
5.1H11 Antibody	5/28/92	TBD	KM 8/21/01
Biotinylated GAM	L0602	NA	
Ultra Avidin-FITC, P/N	0106L235	NA	
IgG_1, isotype control	M049485	NA	✓

Final Product Lot No.	% of Cells Staining Positive for 5.1H11 Stain by FACS	Operator Initials and Date
M1029 Final Product	61.6%	KM 8/24/01
	NA	
NA	KM 8/24/01	

Comments: N/A 8/24/01

Reviewed By: [signature] Date: 8/24/01

QF271-1
Effective Date: DEC 15 2000

Exhibit B

OFFICIAL COPY **DIACRIN, INC.** MASTER COPY

Fusion Assay Data Record

Performed By: JZ Date: 8/23/01

Final Product Lot No.	Final Growth Media Lot #	Fusion Medium Lot #	Date Seeded	Date Switched to Fusion Medium	Date* Myotubes observed	Performed By:	Date:	Fusion Pass/Fail
ch1 Myo29 (8/9/01)	8/9/01	6/8/01	8/10/01	8/13/01	8/15/01		8/15/01	Pass
ch2 Myo29 (8/13/01)	8/9/01	6/8/01	8/13/01	8/14/01	8/16/01		8/16/01	Pass
ch3 Myo29 (8/20/01)	8/20/01 8/21/01	6/8/01	8/20/01	8/21/01	8/23/01		8/23/01	Pass
ch4 Myo29 (8/22/01)	8/9/01	6/8/01	8/21/01	8/22/01	8/24/01		8/24/01	Pass
final harvest Myo29	8/9/01	6/8/01	8/23/01	8/24/01	8/27/01		8/27/01	Pass
N/A								

* Fusion starts 24-48 hours after switching to fusion medium and is complete 72 hours after switching to fusion medium.

Reviewed By: [signature] Date: 8/27/01

QF270-0

Effective Date:

Exhibit C

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JUN 3 2003

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8. The average $|\Delta F/F| = 0.43$, where $\Delta F = F_{ap} - F_{aa}$, compared with 0.082 for maltopentaose alone [E. Goldsmith, S. Sprang, R. J. Fletterick, *J. Mol. Biol.* 156, 411 (1982); E. J. Goldsmith and R. J. Fletterick, *J. Pure Appl. Chem.* 55, 577 (1983)]. Maltopentaose alone induced global changes that are highly correlated with but much reduced in magnitude (25%) from the changes induced by both ligands and caused virtually none of the specific changes induced by phosphate in active site.
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9 January 1989; accepted 28 April 1989

5-Bromo-2′-Deoxyuridine Blocks Myogenesis by Extinguishing Expression of MyoD1

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The pyrimidine analog 5-bromodeoxyuridine (BUdR) competes with thymidine for incorporation into DNA. Substitution of BUdR for thymidine does not significantly affect cell viability but does block cell differentiation in many different lineages. BUdR substitution in a mouse myoblast line blocked myogenic differentiation and extinguished the expression of the myogenic determination gene MyoD1. Forced expression of MyoD1 from a transfected expression vector in a BUdR-substituted myoblast overcame the block to differentiation imposed by BUdR. Activation of BUdR-substituted muscle structural genes and apparently normal differentiation were observed in transfected myoblasts. This shows that BUdR blocks myogenesis at the level of a myogenic regulatory gene, possibly MyoD1, not by directly inhibiting the activation of muscle structural genes. It is consistent with the idea that BUdR selectively blocks a class of regulatory genes, each member of which is important for the development of a different cell lineage.

THE SUBSTITUTION OF BUdR FOR thymidine in DNA has the effect of blocking the expression of the differentiated phenotype in many different cell lineages without significantly altering the general, or household, functions of a cell or cell viability (*1–4*). The ability of BUdR to block differentiation is directly related to the degree of DNA substitution, and, in general, the effect is reversible when cells are cultured in the absence of BUdR and the analog is replaced by thymidine during DNA replication (*5*). Therefore, BUdR is not acting as a mutagen, but is reversibly blocking the differentiation program of a wide variety of cell types in a manner dependent on BUdR incorporation into DNA (*6, 7*).

Although the mechanism by which BUdR blocks differentiation is not known, two types of experiments have suggested that BUdR inhibits differentiation by influencing a small number of regulatory loci: (i) During chick erythropoiesis, increasing concentrations of BUdR result in the production of progressively fewer erythrocytes; however, the erythrocytes that are formed, even at high levels of BUdR substitution, are normal in every way tested (*3*). This all-or-none effect of BUdR inhibition, together with the observation that the dose-response curve was consistent with only a few targets per cell (*8*), suggested that the primary effect of BUdR is the inactivation of a regulatory gene, or master switch, for erythropoiesis (*9*). (ii) In primary chick myoblast cultures blocked from differentiation by a single round of DNA replication in BUdR, the kinetics of myotube differentiation after removal of BUdR and resubstitution with thymidine suggested that the BUdR-sensitive target or targets segregated with only one pair of chromosomes (*10*).

Recently, we have identified a nuclear protein, MyoD1, which can activate the myogenic program in many, but not all, cell types (*11*). The cDNA for this protein was isolated by subtractive hybridization of cDNA from a myoblast line derived from

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the mouse fibroblast cell line C3H10T1/2 (10T1/2 cells) by treatment with 5-azacytidine. 5-Azacytidine is thought to activate the myogenic program in 10T1/2 cells by stable demethylation of a myogenic locus, leading to a heritable myogenic phenotype (*12*). Myoblast cell lines derived from 10T1/2 cells by treatment with 5-azacytidine (aza-myoblasts) express MyoD1, whereas the parental 10T1/2 cells do not (*11*). It is not yet clear whether MyoD1 is the locus responding to 5-azacytidine directly or is responding to a trans-activating factor that is expressed after 5-azacytidine treatment. It is clear, however, that MyoD1 is a nuclear protein (*13*), and when the MyoD1 cDNA is expressed in either serum-starved 10T1/2 cells or a variety of other cell types; many, if not all, of the muscle structural genes are activated (*11*). In this regard, MyoD1 is a master regulatory gene for myogenesis.

To analyze the effect of BUdR substitution on the expression of MyoD1 RNA and RNA of other muscle-specific genes, we plated 10T1/2 cells and aza-myoblasts at low density in growth medium [Dulbecco's modified essential medium (DMEM) supplemented with 15% fetal calf serum and 10 μM deoxycytidine] with or without the addition of 5 μM BUdR (*14*). After 4 days, a time sufficient for most of the cells to have incorporated BUdR into their DNA, parallel plates of cells were harvested for RNA analysis or were switched to differentiation medium (DMEM supplemented with 2% horse serum and 10 μM deoxycytidine) for an additional 4 days while the level of BUdR supplementation was maintained as before. In the absence of BUdR, the aza-myoblasts expressed MyoD1 mRNA in growth medium (Fig. 1, lanes 5 and 9) and when transferred to differentiation medium fused to form myotubes and initiated the expression of myosin heavy chain, myosin light chain 1/3, and desmin (Fig. 1, lanes 7 and 11). In the presence of BUdR, MyoD1 expression was significantly attenuated, and the cells neither fused nor initiated expression of the muscle structural genes when placed in differentiation medium (Fig. 1, lanes 6, 8, 10, and 12).

If BUdR acted to block differentiation through a MyoD1-dependent mechanism, then forced expression of MyoD1 in BUdR-substituted cells might bypass the BUdR blockade. Stable myogenic clones can be derived from 10T1/2 cells by transfection of a plasmid that contains the MyoD1 cDNA driven by a viral long terminal repeat (LTR) (*11*). Although these cells (10T1/2-LTR-MyoD1 cells) presumably lack some or all of the regulatory information that controls expression of the MyoD1 gene in aza-myoblasts, they show many of the characteristics of aza-myoblasts. In growth medium, these cells replicate and express MyoD1 mRNA (Fig. 1, lane 13), and, when shifted to differentiation medium, they fuse to form myotubes (*11*) and initiate the expression of muscle structural genes (Fig. 1, lane 15). In contrast to aza-myoblasts, BUdR-substituted 10T1/2-LTR-MyoD1 cells continue to express MyoD1 mRNA (Fig. 1, lanes 14 and 16) and protein (*15*), presumably because of an insensitivity of the LTR to the inhibitory effect of BUdR. When cultured in differentiation medium the BUdR-substituted 10T1/2-LTR-MyoD1 cells will fuse (*15*) and express muscle structural genes (Fig. 1, lane 16), showing that MyoD1 can activate muscle structural genes even in a BUdR-substituted cell. Similar results are obtained with concentrations of 50 μM BUdR (*15*).

A second analysis of the ability of LTR-driven MyoD1 to bypass the block to differentiation imposed by BUdR substitution was undertaken in a transient transfection assay. Aza-myoblasts or 10T1/2 cells, both of which had BUdR substituted for thymidine, were transfected with either the MyoD1 expression vehicle or with the expression vector lacking the MyoD1 sequence as a control. After transfection, the cells were cultured in differentiation medium for 2 days and then processed for immunohistochemical localization of myosin heavy chain and desmin. BUdR-substituted 10T1/2 cells and aza-myoblasts had roughly the same frequency of myosin- and desmin-positive cells after transfection with the MyoD1 expression vehicle as did unsubstituted 10T1/2 cells after transfection (Table 1). Again, these results are consistent with the conclusion that LTR-driven MyoD1 expression in a BUdR-substituted myoblast is sufficient to activate the terminal myogenic program. The very low level of myogenesis in the substituted aza-myoblasts transfected with the expression vector alone is similar to the level seen in BUdR-substituted aza-myoblasts without transfection (*15*). This similarity demonstrates that the bypass of the block to differentiation is dependent on the expression of transfected MyoD1, not secondary to the transfection process alone.

Since the muscle structural genes remained responsive to trans-activation in BUdR-substituted cells, we wanted to know if the MyoD1 gene could also respond to regulatory factors in a BUdR-substituted cell. We have shown that expression of an LTR-driven MyoD1 construct in 10T1/2 cells will activate the endogenous MyoD1 gene (*16*). The transcript from the LTR-

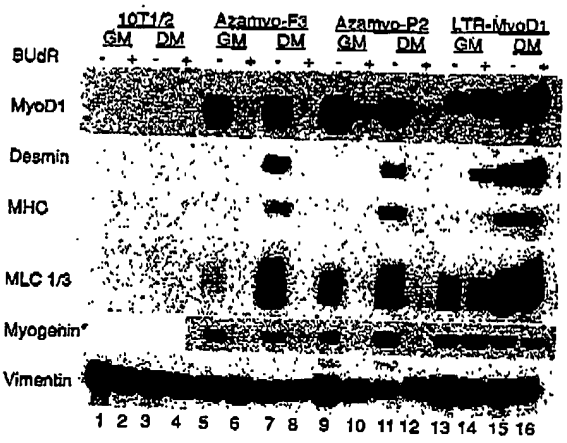

Fig. 1. Inhibition of MyoD1 and muscle-specific gene expression by BUdR substitution. The figure is a composite RNA blot analysis showing expression of muscle-specific genes in unsubstituted and BUdR-substituted cells. 10T1/2 cells, two different clones of aza-myoblasts (Azamyo-F3 and Azamyo-P2), and a clone of 10T1/2-LTR-MyoD1 cells were plated at low density in growth medium with (+) or without (−) supplementation with 5 μM BUdR. Cultures were refed every 2 days. After 4 days, when cultures achieved confluence, one set was harvested for RNA (GM) and a second set shifted to differentiation medium (DM) for an additional 4 days while maintaining the previous level of BUdR supplementation. RNA was recovered by rinsing the cultures with tris-buffered saline, followed by cell lysis and brief sonication in 6*M* urea and 3*M* LiCl (*32*). The RNA was pelleted by centrifugation after incubation at −20°C overnight. The RNA pellet was resuspended in 10 m*M* tris (pH 7.5), 5 m*M* EDTA, and 0.1% SDS, phenol-chloroform was extracted, and ethanol was precipated. Five micrograms of total RNA was loaded on each lane of a 1.5% agarose gel containing 6.7% formaldehyde. Ethidium bromide staining of parallel gels was performed to check the integrity and amount of RNA. Gels were treated for 40 min with 50 m*M* NaOH, 10 m*M* NaCl, neutralized for 40 min in 100 m*M* tris (pH 7.4), 20× SSC (standard saline citrate) and transferred overnight to GeneScreen (DuPont Biotechnology Systems) in 20× SSC. RNA was cross-linked by exposure to ultraviolet light and then baked dry. Blots were hybridized in Stark's solution with 1% SDS at 42°C for 1 hour and then hybridized overnight at 42°C in Stark's solution with 10% dextran and 1% SDS. Probe (5 × 10^5 to 10 × 10^6 dpm) was used for each blot. Blots were washed in 0.4× SSC at 65°C and exposed at −70°C. The probes MyoD1, myosin heavy chain (MHC), and myosin light chain 1/3 (MLC 1/3) were described previously (*11*). *A separate RNA blot was probed with myogenin and did not contain 10T1/2 samples.

MyoD1 construct lacks ~40 nucleotides from its 5′ end, relative to the major start site of genomic transcription, and we can therefore analyze the relative levels of genomic MyoD1 RNA and LTR-MyoD1 RNA by using a ribonuclease protection assay. Consistent with our previous RNA blot analyses (Fig. 1, lanes 1 to 4), we did not detect any MyoD1 in 10T1/2 cells, whereas two fragments of approximately 135 and 155 nucleotides were protected in aza-myoblasts (Fig. 2, lane 1), presumably representing two different start sites of transcription. BUdR substitution extinguishes the expression of the protected RNA (Fig. 2, lane 2) and when we allowed the aza-myoblasts to replicate for several generations in the absence of BUdR, they reexpressed the MyoD1 transcript (Fig. 2, lane 3). A smaller protected fragment representing the shorter LTR-driven transcript (95 nucleotides) was seen in 10T1/2-LTR-MyoD1 cells (Fig. 2, lane 4). In addition, the presence of both the 135- and 155-nucleotide fragments indicates that these cells have activated transcription of their endogenous MyoD1 gene. In unsubstituted 10T1/2-LTR-MyoD1 cells, genomic MyoD1 transcripts were as abundant, if not more abundant, than the LTR-driven transcripts. After substitution with BUdR, however, the amount of genomic transcripts decreased and the amount of LTR-driven transcripts increased (Fig. 2, lane 5). Therefore, in contrast to the muscle structural genes that can be activated to nearly normal levels in BUdR-substituted cells by the forced expression of MyoD1 (as judged by RNA blot analysis, see Fig. 1), the ability of the MyoD1 gene to respond to autoactivation is attenuated in BUdR-substituted cells. It should be noted that genomic MyoD1 expression is not entirely extinguished by BUdR substitution in either aza-myoblasts or 10T1/2-LTR-MyoD1 cells, and we do not currently know whether this represents normal levels of expression in a small fraction of cells or continued low levels of expression in all the cells. The failure of LTR-MyoD1 to fully activate endogenous MyoD1 expression after BUdR substitution may reflect either a cis inhibition of the MyoD1 gene to respond to MyoD1-mediated activation or the loss of a trans-activating factor that normally cooperates with MyoD1 in activating the gene.

Fig. 2. Ribonuclease (RNase) protection assay showing inhibition of genomic MyoD1 expression in BUdR-substituted aza-myoblasts and 10T1/2-LTR-MyoD1 cells. Lane 1, aza-myoblasts; lane 2, aza-myoblasts cultured in 5 μ*M* BUdR for 4 days; lane 3, aza-myoblasts cultured in 5 μ*M* BUdR for 4 days and then an additional 7 days in medium without BUdR supplementation; lane 4, 10T1/2-LTR-MyoD1 cells; lane 5, 10T1/2-LTR-MyoD1 cells cultured in 5 μ*M* BUdR for 4 days; and lane 6, markers. Genomic sequences representing the 5′ end of the MyoD1 gene were cloned into the Bluescribe vector (Stratagene) and T7 polymerase–generated transcripts were made in the presence of [α-^{32}P]CTP. The RNA probe corresponded to genomic positions −592 through +95 relative to the major start site of transcription, as determined by primer extension (*15*). Probe (10^5 cpm) was hybridized overnight at 65°C to 10 μg of total RNA in 20 μl of hybridization buffer [40 m*M* Pipes (1,4-piperazinediethanesulfonic acid, *p*H 6.4), 0.4*M* NaCl, 1 m*M* EDTA, and 80% formamide]. The next day 250 μl of digestion buffer [10 m*M* tris (*p*H 7.4), 300 m*M* NaCl, and 5 m*M* EDTA] with RNase A (5 μg/ml) and RNase T1 (40 U/ml) was added, and the samples were incubated at 30°C for 30 min. The samples were brought to 0.5% SDS and proteinase K (0.2 mg/ml) and incubated at 37°C for 15 min. Carrier transfer RNA was added and the samples were extracted with phenol-chloroform and precipitated with ethanol. The protection products were separated by electrophoresis on an 8% acrylamide and 7*M* urea gel.

These results suggest that incorporation of BUdR in the muscle structural genes may not contribute significantly to the ability of BUdR to block differentiation. Instead, the data lead to the conclusion that BUdR is blocking MyoD1 expression, either directly or indirectly, and the absence of MyoD1 precludes the expression of the myogenic program in these cells. If this is the case, then an unsubstituted muscle structural gene should be inactive in a BUdR-substituted cell because of the absence of MyoD1. To test this idea, we used plasmid constructs containing the reporter gene chloramphenicol acetyltransferase (CAT) driven by the upstream activation sequences from either desmin (DES-CAT) (*17*) or muscle creatine kinase (MCK-CAT) (*18*). Both of these constructs are inactive in 10T1/2 cells (*15*) but are active in differentiated aza-myotubes. These constructs, and control CAT constructs containing the Moloney sarcoma virus LTR (MSV-CAT) (*19*) or the simian virus 40 (SV40) early transcription region (SV2-CAT) (*20*), were introduced into aza-myoblasts or BUdR-substituted aza-myoblasts by electroporation. Electroporation was used because of the observation that standard transfection protocols in which calcium phosphate precipitation is used inhibited MyoD1 protein expression as assayed by immunohistochemistry (*15*). Two days after electroporation, the cells were transferred to differentiation medium and, after an additional 2 days, were harvested for CAT assays. The activity of both MCK-CAT and DES-CAT was diminished in the BUdR-substituted aza-myoblasts compared to the unsubstituted cells (Fig. 3). Co-electroporation with a MyoD1 expression plasmid (LTR-MyoD1) restored the activity

Table 1. The number of cells expressing myosin or desmin after transfection with a MyoD1 expression vector or a control vector. 10T1/2 cells and aza-myoblasts (Aza-myo) were cultured for 4 days in growth medium with (+BUdR) or without (−BUdR) supplementation with 5 μ*M* BUdR. The cells were transfected with 5 μg of either the MyoD1 expression vector (MSV-LTR driving the MyoD1 cDNA) or the expression vector lacking the MyoD1 insert in a calcium phosphate precipitation. The next day the cells were placed in differentiation medium and 2 days later were fixed in 2% formaldehyde for 7 min, permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min and double-labeled with a rabbit antiserum to desmin (*30*) and a mouse monoclonal antibody to myosin heavy chain (*31*), followed by a rhodamine-conjugated donkey antibody to rabbit immunoglobulin G and a fluorescein-conjugated goat antibody to mouse IgG. The number of desmin- or myosin-positive cells in a standard area (~1% of a 60-mm tissue culture dish, an area containing ~3000 cells) of each dish was counted. ND, not done.

Vector	Number of cells expressing			
	Myosin		Desmin	
	10T1/2	Aza-myo	10T1/2	Aza-myo
−BUdR				
Control	0	ND	0	ND
MyoD1	239	ND	147	ND
+BUdR				
Control	0	30*	0	45*
MyoD1	309	391	415	376

*Since nearly all of the ~3000 cells in the counted area would differentiate in the absence of BUdR, the BUdR substitution has blocked myogenesis in roughly 98% of the aza-myoblasts.

of both MCK-CAT and DES-CAT in BUdR-substituted cells. Although we have not demonstrated that the transfected plasmids have not replicated, since they do not contain the elements necessary to support replication (*21*), we believe that we are assaying the activity of unsubstituted regulatory sequences. Effects of both BUdR substitution and MyoD1 expression on the control plasmids, MSV-CAT and SV2-CAT, were also observed (Fig. 2). Our results support the conclusion that the inactivity of muscle-specific terminal differentiation genes in a BUdR-substituted aza-myoblast is secondary to the lack of MyoD1 and not dependent on substitution of the structural gene itself. Billeter *et al.* (*22*) have similarly shown that the regulatory sequences of the myosin light chain 1/3 gene are inactive when transfected into BUdR-substituted myoblasts, suggesting that the BUdR-mediated inhibition of myogenesis effects a trans-acting regulator of this gene.

MyoD1 belongs to a family of regulatory genes that share a region containing a high degree of similarity to a region present in the Myc family of proteins (*11*). Two other members of this family are involved in the regulation of skeletal myogenesis, myogenin (*23*) and Myf 5 (*24*), and both can activate the myogenic program when transfected into 10T1/2 cells. Myogenin is not expressed in 10T1/2 cells (*16, 23*) but is expressed in both aza-myoblasts and 10T1/2-LTR-MyoD1 cells (Fig. 1, lanes 5, 7, 9, and 11). BUdR substitution extinguishes the expression of myogenin in aza-myoblasts (Fig. 1, lanes 6, 8, 10, and 12), whereas myogenin is not inhibited in BUdR-substituted 10T1/2-LTR-MyoD1 cells (Fig. 1, lanes 14 and 16). These data suggest that BUdR acts by extinguishing the maintenance of expression of myogenic regulatory genes and that the expression of MyoD1 is sufficient to bypass this blockade and reactivate at least one other myogenic regulatory gene. We cannot conclude that BUdR does not have an independent effect on myogenin expression, since it is possible that the maintenance of the determined myogenic state relies on the interaction of MyoD1, myogenin, and potentially other regulatory genes in an autoregulatory system, in which altering the expression of any member could affect the expression of the others. In this regard, we should note that (i) forced expression of myogenin in 10T1/2 cells will activate MyoD1 expression (*16*), but we do not know if this activation is inhibited by BUdR substitution; and (ii) the differentiation of rat L6 myoblasts, which express myogenin but not MyoD1, is inhibited by BUdR (*7*).

The ability of BUdR to reversibly inhibit differentiation in many different cell lineages without significantly affecting the household functions of the cell was one of the observations used by Holtzer and colleagues to postulate the existence of a family of master regulatory genes whose activity could be selectively blocked (*9*). Our results show that BUdR substitution in aza-myoblast DNA extinguishes the expression of MyoD1, whereas the muscle structural genes remain responsive to activation by muscle regulatory factors. We have not yet determined whether BUdR inhibits MyoD1 expression by a *cis* or trans mechanism. One possibility is that BUdR incorporation alters gene expression by changing the binding affinity of transcriptional activators or inhibitors, as has been shown for the *lac* repressor (*25*). If this occurs uniformly for both constitutive and tissue-specific genes, resulting in small alterations of the binding affinities of DNA binding proteins, then the particular sensitivity of such regulatory genes as MyoD1 to BUdR substitution would still need to be explained. We propose that amplification of expression by positive autoregulation could make MyoD1 particularly sensitive to slight degrees of inhibition that could lead to dampening of the feedback loop and a loss of amplified gene activity. It is possible that many different cell lineages use positive autoregulatory feedback circuits to amplify expression of genes that control development, such as has been shown not only for MyoD1 (*16*), but also for some of the *Drosophila* homeobox genes (*26*). BUdR substitution could possibly dampen these positive feedback loops, leading to a selective inhibition of this subset of regulatory genes. Since BUdR inhibition is reversible, the BUdR-repressed cells must retain a memory of their committed myogenic potential. The fact that MyoD1 *is* inhibited in BUdR-substituted aza-myoblasts suggests that the BUdR-resistant memory resides at a genetic locus that is upstream of MyoD1 in the regulatory pathway. Alternatively, some change at the MyoD1 gene, for example, demethylation of a regulatory sequence, might be responsible for myogenic memory.

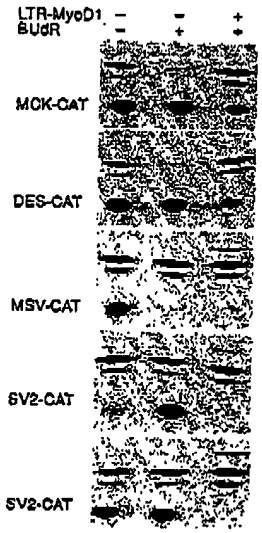

Fig. 3. Activity of muscle-specific regulatory sequences in BUdR substituted aza-myoblasts (first four assays) or 10T1/2 cells (last assay). Cells were cultured at low density in growth medium either with (BUdR +) or without (BUdR −) supplementation with 5 μ*M* BUdR for 4 days before electroporation. Approximately 10^7 cells were suspended in 800 μl of FBS (*p*H 7.4) with 20 μg of the CAT vector and 20 μg of either the MyoD1 expression vector (LTR-MyoD1 +) or the expression vehicle lacking the MyoD1 insert (LTR-MyoD1 −). Electroporation was performed with a Bio-Rad Gene Pulser. Cells were plated in growth medium overnight for 2 days and then switched to differentiation medium for 2 days, at their previous level of BUdR substitution. Cultures were rinsed with PBS, scraped into ~300 μl of PBS, sonicated, and centrifuged. Equivalent amounts of protein were used for CAT assays for each construct. CAT assays were performed as described (*33*).

A second explanation for the effect of BUdR is that a single BUdR-responsive gene is involved in regulating the expression of MyoD1 and other "master regulatory genes." For example, BUdR substitution could result in the overproduction of an active oncogene that suppresses the expression of MyoD1 and related genes. Expression of activated *ras* in C2C12 myoblasts will also both block differentiation (*27*) and block the expression of MyoD1 (*28*). In these cells, as in BUdR-blocked aza-myoblasts, expression of MyoD1 will bypass the *ras* blockade (*28*). A similar result is observed when *c-fos* is expressed constitutively in aza-myoblasts (*28*). Moreover, a number of nondifferentiating variants of aza-myoblasts lack MyoD1 expression (*11*) but can be induced to differentiate by the LTR-driven MyoD1 expression vector (*29*). Thus, inhibition of MyoD1 seems to be a common pathway for the inactivation of the myogenic program. We hope that our current efforts to characterize MyoD1 regulatory elements will help us to determine if BUdR is acting in *cis* or altering the production of a trans-acting factor.

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17. The p3.4Des-CAT construct was generously supplied by Dr. Hans Bloemendal [F. R. Pieper, R. L. Slobbe, F. C. S. Ramaekers, H. T. Cuypers, H. Bloemendal, *EMBO J.* 6, 3611 (1987)].
18. The MCK-CAT construct was the gift of S. Hauschka [J. B. Jaynes, J. E. Johnson, J. N. Buskin, C. L. Gartside, S. Hauschka, *Mol. Cell. Biol.* 8, 62 (1988)].
19. The MSV-CAT consists of the MSV LTR driving a CAT gene and was the gift of Hazel Sive.
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"The candidates for the cell biologist job are here and, Dr. Francis, I think one of them has a big edge."

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